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RAPID COMMUNICATION

Nitric oxide donor-mediated inhibition of phosphorylation shows that light-mediated degradation of photosystem II D1 protein and phosphorylation are not tightly linked

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Abstract An outcome of the photochemistry during oxygenic photosynthesis is the rapid turn over of the D1 protein in the light compared to the other proteins of the photosystem II (PS II) reaction center. D1 is a major factor of PS II instability and its replacement a primary event of the PS II repair cycle. D1 also undergoes redox-dependent phosphorylation prior to its degradation. Although it has been suggested that phosphorylation modulates D1 metabolism, reversible D1 phosphorylation was reported not to be essential for PS II repair in *Arabidopsis*. Thus, the involvement of phosphorylation in D1 degradation is controversial. We show here that nitric oxide donors inhibit in vivo phosphorylation of the D1 protein in *Spirodela* without inhibiting degradation of the protein. Thus, D1 phosphorylation is not tightly linked to D1 degradation in the intact plant.

Keywords Chloroplast · Reaction center proteins · *Spirodela* · Duckweeds · D1 phosphorylation · Nitric oxide

Abbreviations

CAM	Crassulacean acid metabolism
NaF	Sodium fluoride
NO	Nitric oxide
PS II	Photosystem II
SIN-1	3-Morpholinosydnonimine
SNOC	*S*-Nitrosocysteine

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Introduction

Much of life on earth is sustained by oxygenic photosynthesis. The process involves four multisubunit membrane–protein complexes: photosystem II (PS II), photosystem I (PS I), cytochrome *b6f* and F-ATPase (Nelson and Yocum 2006). PS II and PS I are intrinsic pigment–protein complexes spatially separated in the chloroplast. PS II catalyzes the oxidation of water and the reduction of plastoquinone (PQ). It contains at its core a reaction center, consisting of the D1 and D2 proteins, α and β subunits of cytochrome b_{559}, the *psb*I gene product and a few low molecular weight polypeptides. The D1–D2 heterodimer binds all the electron carriers and cofactors necessary for electron transport: P680 (a chlorophyll *a* dimer), pheophytin *a*, a non-heme-iron, β-carotene, the electron donor tyrosine, and the electron acceptor PQ (Nanba and Satoh 1987; Mattoo et al. 1989). One of the major outcomes of light-mediated photochemistry is that, in the light, D1 protein is rapidly turned over compared to the other proteins of the reaction center. Therefore, D1 is a major factor of PS II instability and its replacement a primary event of the PS II repair cycle (Yokthongwattana and Melis 2006; Edelman and Mattoo 2008). Rapid turnover of D1 is conserved throughout evolution among oxygen evolving species (Mattoo et al. 1989; Yokthongwattana and Melis 2006; Edelman and Mattoo 2008) but the physiological significance of this is not well understood.

D1 is a target of at least five post-translational modifications during its life cycle, including N-acetylation, palmitoylation and phosphorylation (Edelman and Mattoo 2008).

One or more of these post-translational modifications could potentially alter protein degradation kinetics. A number of suggestions have been made for the role of D1 phosphorylation, including: turning off PS II (or rerouting electron transport) in order to protect the photosystem from damage caused by high light intensity (Yokthongwattana and Melis 2006), downregulating D1 metabolism (Rintamäki et al. 1995a) by controlling the timing of its proteolysis (Aro et al. 2005), providing protection against photoinhibition (Harrison and Allen 1991), preventing disassembly of PS II (Aro et al. 1992), or promoting dissociation of some proteins from the core (Giardi 1993) under photoinhibitory conditions. However, in *Spirodela*, where light-dependent phosphorylation of D1 is regulated by a circadian clock (Booij-James et al. 2002), the greatest amount of phosphorylation occurs hours before maximal light intensity (Booij-James et al. 2002), and at intensities well below those saturating for photosynthesis (Jansen et al. 1996) or initiation of photoinhibition (Jansen et al. 1999). In addition, using a molecular genetics approach, Bonardi et al. (2005) reported that reversible D1 phosphorylation is not essential for PS II repair in *Arabidopsis* (also see http://www.mcponline.org/cgi/eletters/5/8/1412). Finally, D1 does not appear to undergo light-dependent phosphorylation in the moss *Ceratodon purpureus* (Rintamaki et al. 1995b) or in cyanobacteria (Allen 1992), although light-dependent rapid D1 degradation occurs in these organisms. Thus, the involvement of phosphorylation in D1 degradation is controversial.

Nitric oxide (NO) is a signaling molecule that plays a role in a variety of cellular processes in animals, and was recently shown to impact a wide range of processes in plants (Arasimowicz and Floryszak-Wieczorek 2007). NO can stimulate or inhibit plant processes, probably as a function of its local concentration and ability to directly interact with cellular reactions and other signals (Arasimowicz and Floryszak-Wieczorek 2007). Quite a number of genes (Parani et al. 2004) and proteins (Lindermayr et al. 2005) that respond to NO have been identified in *Arabidopsis*. Proteins associated with carbon, nitrogen and sulfur metabolism, cytoskeleton, stress and photosynthesis were likewise identified in the soluble *S*-nitrosoproteome of a crassulacean acid metabolism (CAM) plant, *Kalanchoe pinnata,* using a biotin-switch assay (Abat et al. 2008). NO-mediated regulation of photosynthesis-related processes and proteins includes: S-nitrosylation of the major chloroplast protein Rubisco (Abat et al. 2008); inhibition of photophosphorylation (Takahashi and Yamasaki 2002); and effects on PS II photochemistry involving Q_A^- reoxidation and steady state photochemical and nonphotochemical quencing (Wodala et al. 2008).

One way to test the dependence of D1 degradation on its phosphorylation is to apply inhibitory agents selective for one and not the other process. NO and NO donors were shown to inhibit phosphorylation reaction catalyzed by protein kinase C (Gopalakrishna et al. 1993) and apoptosis signal-regulating kinase 1 (Park et al. 2000). We chose *S*-nitrosocysteine (SNOC) (Ignarro 1990; Lei et al. 1992) and 3-morpholinosydnonimine (SIN-1) (Osakada et al. 2003) as NO donors to determine their in vivo effect on light-dependent phosphorylation of PS II proteins, including D1. We show here that NO donors target and inhibit in vivo phosphorylation of most PS II proteins without significantly affecting de novo protein synthesis. Using an NO donor to inhibit phosphorylation, pulse-chase experiments were performed to show that protein phosphorylation and D1 degradation are not tightly linked.

Materials and methods

Plant material and thylakoid membrane preparation

Our model plant for in vivo studies is *S. oligorrhiza,* a small (1–3 mm) aquatic angiosperm that is particularly suited for investigating in vivo dynamics of chloroplast proteins due to its ability to rapidly uptake nutrients as well as radiolabeled substrates (summarized in Edelman and Mattoo 2008). Axenic cultures of *S. oligorrhiza* were grown phototrophically at 25°C and 30 μmol m^{-2} s^{-1} cool white fluorescent light on half-strength Huntner's medium containing 0.5% (w/v) sucrose (Posner 1967). For pulse-labeling or pulse-chase experiments, the plants were transferred to a medium lacking sucrose for at least 48 h before each experiment. Thylakoids were prepared as previously described (Booij-James et al. 2002) in the presence of 100 mM NaF (to inhibit phosphatase activity during extraction) and suspended in buffer A (10 mM Tricine–NaOH, pH 7.8, 100 mM sorbitol, 10 mM $MgCl_2$, 10 mM NaCl) such that the chlorophyll concentration was greater than 250 μg ml^{-1}. Chlorophyll concentrations were determined in 80% acetone (Arnon 1949).

In vivo pulse-labeling experiments

Spirodela plants transferred to medium lacking sucrose and phosphate and maintained under fluorescent lighting at 50 μmol m^{-2} s^{-1} were pulse-labeled for 3 h with 0.5 mCi ml^{-1} of [^{32}P]orthophosphate, or for 1 h with 0.1 mCi ml^{-1} of [^{35}S]methionine (Elich et al. 1992; Booij-James et al. 2002). The effects of NO donors, SNOC (Ignarro 1990; Lei et al. 1992) and 3-morpholinosydnonimine (SIN-1) (Osakada et al. 2003), were carried out at 1 mM final concentration. SNOC was prepared according to (Lei et al. 1992). SIN-1 was purchased from Alexis Biochemicals. The plants were subsequently washed three times with ice-cold H_2O, collected on dry ice, and stored at −80°C until thylakoids were isolated.

Electrophoresis and protein identification

Thylakoid proteins were solubilized in 1× SDS sample buffer for 1 h at room temperature and separated by SDS-PAGE on 10–20% acrylamide gradient gels (Elich et al. 1992; Booij-James et al. 2002). The samples were loaded on an equal chlorophyll basis (1–2 µg of chlorophyll per lane). The gels were stained with Coomassie Blue R-250 and autoradiographs were quantified. The identity and migration positions of phosphorylated forms of D1, D2, CP43 and LHCII proteins on gradient gels have been previously established for *Spirodela* (Elich et al. 1992, 1993).

Results

Nitric oxide inhibits phosphorylation of D1, D2 and LHCII

Photoautotrophic *Spirodela* plants were incubated with [^{32}P]orthophosphate for 1 h in the light in the absence and presence of 1 mM NO donors, SNOC and 3-morpholino-sydnonimine (SIN-1). As previously shown (Elich et al. 1992, 1993, 1997), robust phosphorylation of D1, D2, CP43-47 and LHCII proteins occurred in the control plants (Fig. 1, lane 1). However, in the presence of both the NO donors, phosphorylation of these proteins was dramatically inhibited except for a relatively lesser effect on D2 (Fig. 1, lanes 2 and 3). A number of other proteins, whose identity remains to be established, were also phosphorylated in the control plants (unmarked bands in Fig. 1, lane 1). Their phosphorylation was also inhibited by NO donors SNOC and SIN-1 (Fig. 1, lanes 2 and 3).

Nitric oxide does not inhibit the synthesis of chloroplast proteins

Nitric oxide donor-mediated inhibition of PS II phosphorylation could be due to inhibition of de novo protein synthesis, as in mammalian cells (Kolpakov et al. 1995). Therefore, we assessed the effects of 1 mM SNOC and SIN-1 on protein synthesis. Photoautotrophic *Spirodela* plants, pre-incubated in the dark for 10 min in the absence or presence of SNOC or SIN-1, were given 0.1 mCi ml^{-1} [^{35}S]methionine in the light (50 µmol m^{-2} s^{-1}) for 60 min. The membrane proteins were isolated and fractionated by SDS-PAGE. The results show that D1 protein synthesis is not impaired by either SNOC or SIN-1, while SIN-1 has a positive effect on the synthesis of LHCII (Fig. 2, compare SNOC and SIN1 lanes with C). Thus, the effect of the NO donors on PS II protein phosphorylation is relatively specific and not due to inhibition of protein synthesis.

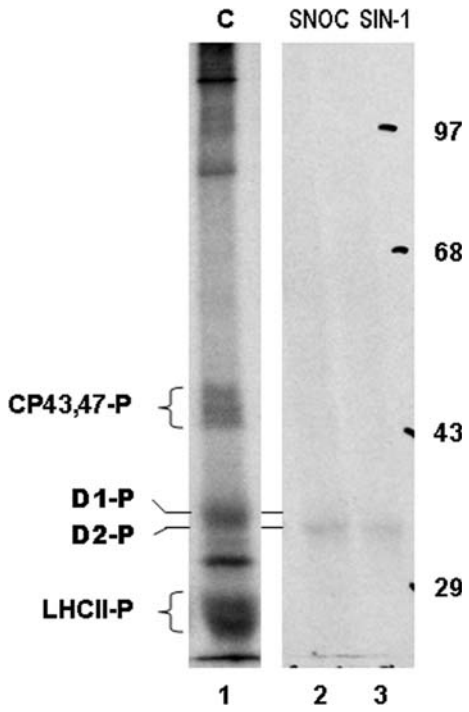

Fig. 1 Nitric oxide donors inhibit in vivo phosphorylation of D1, LHCII and CP43-47 proteins. Photoautotrophic *Spirodela* plants were pre-incubated in the dark for 10 min in the absence (*C*) (*lane 1*) or presence of 1 mM SNOC (*lane 2*) or 1 mM SIN-1 (*lane 3*), then labeled in the light (50 µmol m^{-2} s^{-1}) with 0.5 mCi ml^{-1} of [^{32}P]orthophosphate for 3 h in 1 ml of mineral medium. The plants were then harvested, put on dry ice and stored at −70°C until used. The positions of CP43-47, D1, D2 and LHCII proteins are indicated. The identity of other phosphorylated proteins (unmarked in *lane 1*) is not known. Samples were loaded on an equal chlorophyll basis (1 µg of chlorophyll per lane)

Effect of SNOC on D1 degradation

We sought to determine if SNOC inhibits D1 degradation. Photoautotrophic *Spirodela* plants were pulse-labeled with [^{35}S]methionine in the light and then chased on half-strength Huntner's medium containing unlabeled methionine with or without 1 mM SNOC for up to 8 h. Since it is recommended that SNOC (in solution) be used within 2 h of preparation (Lei et al. 1992), the appropriate chase medium was replenished with SNOC every hour during the chase period. Data from a typical pulse-chase experiment are shown in Fig. 3a. The experiments were repeated several times and radiolabeled D1 protein was quantified and normalized. Quantification (mean ± SD; $n = 4$) of D1 protein degradation is presented in Fig. 3b. The results indicate that SNOC did not inhibit D1 degradation, if anything it slightly increased it (Fig. 3b, compare Control and SNOC lanes). A protein band whose position is marked by a star (Fig. 3a, lane 4) and which appears transiently in the thylakoid fraction from samples incubated with SNOC has previously been identified as the oxidized form of large

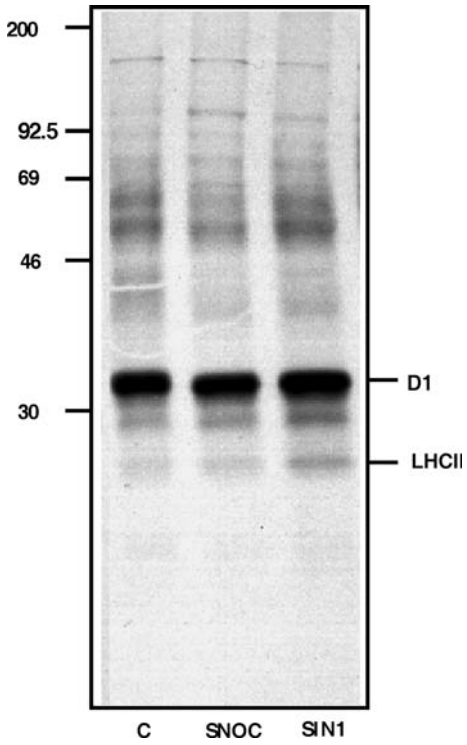

Fig. 2 Nitric oxide donors do not impact D1 synthesis. Photoautotrophic *Spirodela* plants were pre-incubated in the dark for 10 min in the absence (*C*) or presence of 1 mM SNOC (lane marked *SNOC*) or 1 mM SIN-1 (lane marked *SIN1*), then labeled in the light (50 µmol m^{-2} s^{-1}) for 60 min with 0.1 mCi ml^{-1} [^{35}S]methionine in 1 ml of mineral medium. The plants were harvested, immediately put on dry ice and then stored at −70°C until used. The positions of D1 and LHCII proteins are indicated

subunit of ribulose1,5-bisphosphate carboxylase/oxygenase (Mehta et al. 1992).

Discussion

We demonstrate that NO donors target redox-regulated phosphorylation of PS II proteins, including D1. Using this property of NO, we show that, irrespective of inhibiting D1 phosphorylation, D1 degradation kinetics were not significantly different from the control after 4 h and actually somewhat increased after 8 h, possibly due to some accumulation of photodamaged D1 upon interference with the normal life cycle of the protein. While the putative function assigned to D1 phosphorylation in the PS II repair process by Aro et al. (1993) is degradational arrest, the results presented here do not support this hypothesis. Moreover, it should be pointed out that in vivo both the level of D1 phosphorylation and the rate of D1 degradation increase with increasing light intensity (Elich et al. 1992). Thus, D1 degradation is not linked to D1 phosphorylation.

NO interacts with proteins containing transition metal ions (haem or non-haem complexes) (Drapier et al. 1991; Le Brun et al. 1997), reacts with sensitive thiol residues causing S-nitrosylation of proteins (Abat et al. 2008), and modifies tyrosine and tryptophan residues in proteins to enable biological nitration (Gow et al. 2004). The finding of membrane-associated oxidized form of Rubisco large subunit (Mehta et al. 1992) in the samples incubated with

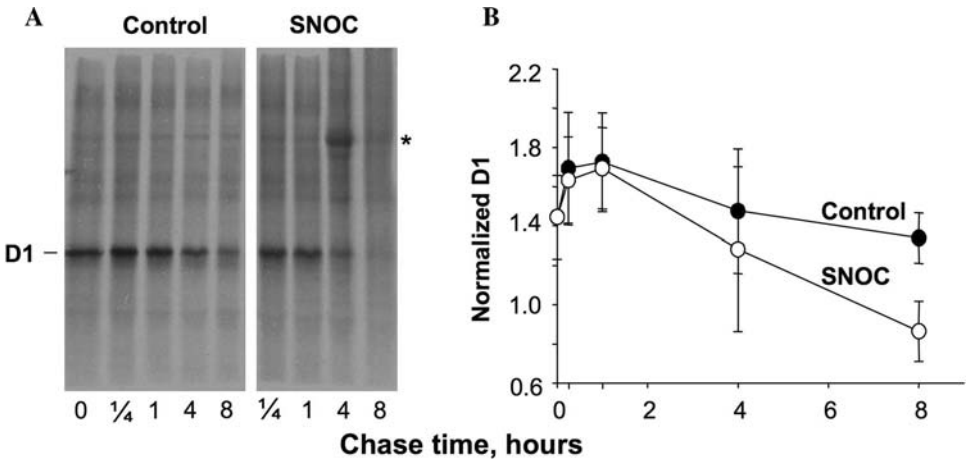

Fig. 3 D1 degradation is not inhibited by SNOC. Photoautotrophic *Spirodela* plants were labeled with 0.1 mCi ml^{-1} [^{35}S]methionine in 1 ml of mineral medium in the light (50 µmol m^{-2} s^{-1}) for 60 min, then chased in the same medium containing 1 mM unlabeled methionine for the times indicated in the absence (*Control*) or presence of 1 mM SNOC. The chase medium in SNOC-treated samples was replaced every hour with fresh addition of SNOC to alleviate problems of SNOC instability. Plants were harvested at the indicated times, put on dry ice and then stored at −70°C until used. The experiments were repeated several times and radiolabeled D1 protein was quantified and normalized. **a** Autoradiogram of a typical SDS-polyacrylamide gel. **b** Quantified data of D1 protein degradation from autoradiograms (mean ± SD; $n = 4$). The protein band whose position is marked by a *star* (*lane 4*) was previously identified as the oxidized form of large subunit of ribulose1,5-bisphosphate carboxylase/oxygenase (Mehta et al. 1992)

SNOC indicates that NO-mediated protein modification in *Spirodela* involves oxidation processes affecting thiol residues. Therefore, being a reactive molecule, NO can impact cellular redox signaling (Foyer and Allen 2003) and thereby mediate inhibition of redox-regulated processes.

A feature presented here is that NO donors target a redox process intimately associated with the chloroplast. We show that NO inhibits redox-dependent phosphorylation of PS II proteins D1, CP43-47, and LHCII; surprisingly D2 is less affected, suggesting that the kinase that phosphorylates D2 is less sensitive to NO donors. Thus, it can be predicted that the protein kinases responsible for phosphorylation of several PS II proteins possess NO-sensitive groups such as transition metals, sensitive thiols, and/or sensitive tyrosine/tryptophan residues.

If phosphorylation is largely disconnected from D1 degradation, as concluded from the results presented here, then what signals D1 turn over in the light and what is the role(s) of D1 phosphorylation? Some lower photosynthetic organisms, including cyanobacteria and algae, do not undergo light-dependent phosphorylation of D1 but possess multiple copies of the *psb*A gene coding for D1, with different D1 isoforms showing a differential response to changing light intensities (Bustos et al. 1990). However, D1 phosphorylation in higher plants (Booij-James et al. 2002), as well as light regulation of *psb*A transcription in cyanobacteria (Liu et al. 1995), is under the control of circadian clocks. We therefore proposed that reversible phosphorylation of D1 in higher plants evolutionarily replaced multiple DNA copies in cyanobacteria as a more energy-efficient substrate for circadian clock regulation of PS II core metabolism (Booij-James et al. 2002; Edelman and Mattoo 2008).

Proteins associated with diverse metabolic processes, including photosynthesis, were highlighted in an analysis of the *S*-nitrosylated proteome members of *Arabidopsis thaliana* (Lindermayr et al. 2005) and *Kalanchoe pinnata,* a CAM plant (Abat et al. 2008). However, these studies were performed on soluble proteins, and no information is available on membrane-associated nitrosylated proteins, which might include protein kinases. Thus, an avenue worth pursuing would be to identify the membrane nitrosylated proteome and ascertain if protein kinases responsible for redox regulation of PS II protein phosphorylation are featured among them.

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